FEBS Letters 581 (2007) 3145–3148

Association of two proteolipids of unknown function with ATP synthase from bovine heart mitochondria

Ruming Chen, Michael J. Runswick, Joe Carroll, Ian M. Fearnley, John E. Walker*

Medical Research Council Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 0XY, United Kingdom

Received 18 May 2007; revised 29 May 2007; accepted 30 May 2007

Available online 8 June 2007

Edited by Peter Brzezinski

Abstract **ATP synthase, or F-ATPase, purified from bovine heart mitochondria in the absence of phospholipids is an assembly of 16 different subunits. In the presence of exogenous phospholipids, two additional hydrophobic proteins, a 6.8 kDa proteolipid and diabetes associated protein in insulin sensitive tissue (DAPIT), were associated with the purified complex, with DAPIT at sub-stoichiometric levels. Both proteins are conserved in vertebrates and invertebrates, but not in fungi, and prokaryotic F-ATPases do not contain orthologues of either of them. Therefore, their roles are likely to be peripheral to the synthesis of ATP.**

Keywords: Mitochondria; ATP synthase; 6.8 kDa proteolipid; DAPIT; Subunit composition

1. Introduction

A number of relatively abundant small hydrophobic proteins, or proteolipids, of unknown function have been characterised by mass spectrometric analyses of the inner membranes of bovine mitochondria [1,2]. They include a 6.8 kDa protein (molecular mass 6834 Da) and diabetes associated protein in insulin sensitive tissue (DAPIT) (or Usmg5, up-regulated during skeletal muscle growth; molecular mass 6303 Da). Their roles are not known. The expression of DAPIT increases during skeletal muscle growth, and was diminished in the same tissue in rats treated with streptozotocin, a drug that induces diabetes [3]. The link between insulin resistance and mitochondria has been confirmed in recent studies of cultured human myotubes [4]. On the basis of tissue specific expression profiles, both 6.8 kDa and DAPIT were placed in an oxidative phosphorylation module [5]. One possibility is that both proteins are hitherto undetected components of one or more of the well known and extensively characterised multi-subunit protein complexes I, II, III and IV, and the ATP synthase (or F-ATPase) that carry out the terminal energy transducing steps of oxidative phosphorylation, leading to the generation of the transmembrane proton-motive force and the synthesis of ATP from ADP and phosphate. To investigate this possibility, as described here, we have employed antibodies against the 6.8 kDa proteolipid and DAPIT to follow their behaviour during the purification of respiratory protein complexes by a combination of ion exchange and gel filtration chromatography. When the complexes were purified in the absence of exogenous phospholipids, both the 6.8 kDa proteolipid or DAPIT followed the F-ATPase in the first purification step, but they were lost in a subsequent step. When phospholipids were included in chromatography buffers, both proteolipids were found in the pure F-ATPase complex. They were found also in independent preparations of F-ATPase prepared by affinity chromatography in the presence of the same phospholipids, but not in their absence.

*Corresponding author. Fax: +44 1223252705.
E-mail address: walker@mrc-dunn.cam.ac.uk (J.E. Walker).

Abbreviations: DAPIT, diabetes associated protein in insulin sensitive tissue; PAGE, polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Materials

Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA), and ion exchange and gel filtration columns from GE Healthcare (Chalfont St. Giles, Bucks, UK). Chicken antibodies to the synthetic peptides RSADKRSKALKASS and EADAQFHFTGIKKY (residues 39–52 and 4–17 of the 6.8 kDa proteolipid and DAPIT, respectively) were raised by AgriSera (Vännäs, Sweden).

2.2. Purification of respiratory protein complexes

Bovine heart mitochondria [6] were extracted with 1% (w/v) *n*-dodecyl-β-D-maltoside [7,8] and the respiratory complexes were fractionated at 4 °C on a Q-Sepharose Hi Trap Q HP column (5 ml) in a buffer A containing 20 mM Tris–HCl pH 8.0, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, 0.05% *n*-dodecyl-β-D-maltoside and 10% (v/v) glycerol either lacking or containing phospholipids [0.01% cardiolipin (w/v), 0.0025% phosphatidylethanolamine and 0.0025% phosphatidylcholine]. Phospholipids were dried down from a chloroform solution under a stream of nitrogen and resuspended in water. The suspension was sonicated, and then the phospholipids were added to buffer to the required concentrations. Proteins were eluted with a gradient of sodium chloride (to 1 M) in the same buffers. Selected fractions were passed through a column of Superose 6 GL 10/300 in buffer A plus 150 mM sodium chloride at a flow rate 0.3 ml/min. F-ATPase was purified independently from bovine heart mitochondria by affinity chromatography in buffers containing the same phospholipids at the same concentrations as described above (M.J. Runswick, J.V. Bason and J.E. Walker, in preparation). ATP hydrolysis activities were measured as described before [7].

2.3. Protein analytical methods

Polyacrylamide gel electrophoresis (PAGE) was performed in acrylamide gradient (12–22%) mini-gels (10 cm × 10 cm) in 0.1% (w/v) SDS [9], and proteins were detected by staining with Coomassie brilliant blue dye. Stained proteins were identified by mass spectrometric analysis of proteolytic digests [2]. Unstained proteins were transferred to (poly)vinylidene difluoride membranes and the 6.8 kDa proteolipid and DAPIT were detected with antisera [2]. Cysteine residues in samples

0014-5793/$32.00
doi:10.1016/j.febslet.2007.05.079

of purified bovine F-ATPase were *S*-carboxymethylated with [^{14}C] 2-iodoacetic acid under denaturing conditions [10] and the subunits were separated by SDS–PAGE. The gels were dried, exposed to a phosphorimage plate, and fluorescence arising from radio-labeled proteins was quantified with a Typhoon 9410 fluorescence imager (GE Healthcare).

3. Results

3.1. Co-purification of the 6.8 kDa proteolipid and DAPIT with F-ATPase

Detergent solubilized proteins from mitochondria were fractionated into six major peaks by anion exchange chromatography in buffer lacking phospholipids (Fig. 1A). Peak 1 contained unbound material, and peaks 2–6, respectively, contained complex II, the F-ATPase complex, complex I, complex IV and complex III, as confirmed by SDS–PAGE. Both the 6.8 kDa proteolipid and DAPIT were detected immunologically in the F-ATPase peak (peak 3 in Fig. 1A). Small amounts were found also in the unbound material (not shown). When pooled fractions from peak 3 were fractionated by gel filtration chromatography in buffers lacking phospholipids, the F-ATPase no longer contained either the 6.8 kDa proteolipid or DAPIT (not shown). Therefore, various concentrations of phospholipids (phosphatidylethanolamine, phosphatidylcholine and cardiolipin) in various combinations, were added to the chromatography buffers, and the experiments were repeated. In the presence of all three phospholipids, the F-ATPase was found in peak 3 (Fig. 1B), whereas complexes I, III and IV co-eluted in peak 4. Both the 6.8 kDa proteolipid and DAPIT remained with F-ATPase containing fractions, and they were also retained in the pure F-ATPase complex obtained by gel filtration chromatography in buffers containing the phospholipids (Fig. 1C). In SDS–PAGE gels, the 6.8 kDa proteolipid and DAPIT were found in the region below subunits F_6, g and A6L (Fig. 2, lane A). Often they both run to the same position (Fig. 2, lane C), but sometimes they are resolved (Fig. 2, lane A). In common with many other hydrophobic proteins, they stain poorly with Coomassie blue dye, although once their position in SDS–PAGE gels was known, staining provided a useful guide to their absence or presence in preparations of ATP synthase.

In preparations of F-ATPase made by affinity chromatography, the 6.8 kDa proteolipid and DAPIT were associated with the enzyme complex. Once again, the association of the 6.8 kDa proteolipid and DAPIT with the F-ATPase complex required the inclusion of phospholipids, and especially cardiolipin, in the chromatography buffer (M.J. Runswick, J.V. Bason and J.E. Walker, in preparation) (Fig. 2, lanes B and C).

3.2. Enzymic properties of affinity purified F-ATPase

In comparison to the enzyme prepared by affinity chromatography in the absence of phospholipids, the enzyme prepared in the same way in the presence of phospholipids has both higher activity (17.2 U/mg compared to 15.4 U/mg) and increased sensitivity to oligomycin (77% inhibition compared with 12%). Addition of phospholipids during the assay to the enzyme prepared in the absence of phospholipids also had the beneficial effect of increasing ATP hydrolysis from 15.4 to 59 U/mg and oligomycin sensitivity from 25% to 47%. However, these effects are lower than the effect of retaining both the 6.8 kDa proteolipid and DAPIT in the complex (together with phospholipids). As the effect of removing phospholipids from the complex may be at least partially irreversible, at present it is not possible to know whether the presence of the 6.8 kDa proteolipid and DAPIT contribute directly to the enhanced activity and sensitivity to oligomycin.

3.3. Stoichiometry of DAPIT in the F-ATPase complex

The bovine F-ATPase complex contains several well-characterized subunits that are present in single copies and also contain a single cysteine residue in their sequences. They include the γ- and ε-subunits, and subunits OSCP, b, d and f [10–13]. DAPIT also contains a single cysteine in its sequence, and so incorporation of radioactivity from [1-^{14}C] iodoacetic acid into the fully denatured proteins provides a means of assessing the molar content of DAPIT relative to other subunits in the purified F-ATPase complex. The ratios of incorporated radioactivity in the bands containing the γ-subunit, subunit b, a band containing unresolved OSCP and d subunits, subunit f, DAPIT and the ε-subunit were 0.54:1.07:1.90:1.0: 0.46:1.08. With the exception of the γ-subunit, the values for subunits b, OSCP and d together, f and the ε-subunit agree with subunit stoichiometries determined previously by the same method [10,11], and in the case of subunits γ, b, d, and ε established by structural analysis [14,15]. The low incorporation of radioactivity into the γ-subunit in similar experiments has been noted before [16]. Therefore, these experiments indicate the presence of substoichiometric amounts of DAPIT in the F-ATPase preparation made in the presence of exogenous phospholipids. Other experiments in progress will be required to confirm this finding, and to establish the content in the complex of the 6.8 kDa proteolipid, which lacks cysteine residues.

4. Discussion

The subunit composition of the F-ATPase complex from bovine heart mitochondria has been studied extensively. The stoichiometries of most of its 16 constituent subunits have been determined [10,11], and the structures of many of them have been established, some of them in subcomplexes, such as the F_1 domain [14] and the peripheral stalk complex [15]. The least well understood part of the complex is its intrinsic membrane domain where protons translocate through the membrane. These proton translocation events drive the rotary mechanism of the enzyme, and couple the proton-motive force mechanically to the synthesis of ATP from ADP and phosphate in the F_1 catalytic domain. The structure of the complex of the membrane associated c-ring and the central stalk that form the rotor has been established in the yeast enzyme [17], but the structure of the c-ring has not been established in the bovine complex. The c-ring rotates against a single a (or ATPase-6) subunit, and together they provide the proton pathway. The membrane domain of the single b-subunit consisting of two membrane spans [12], forms part of the stator and is probably associated with a-subunit [18]. The arrangement and functions of the remaining subunits in the membrane domain are less clear. Each of subunits e, f, g and A6L has a single transmembrane span, but how they are arranged in relation to each other and to subunits a, b and c is not known.

The association of these so-called "minor" subunits with the F-ATPase complex has been studied in a wide range of relatively mild detergents, and under some conditions subunits e

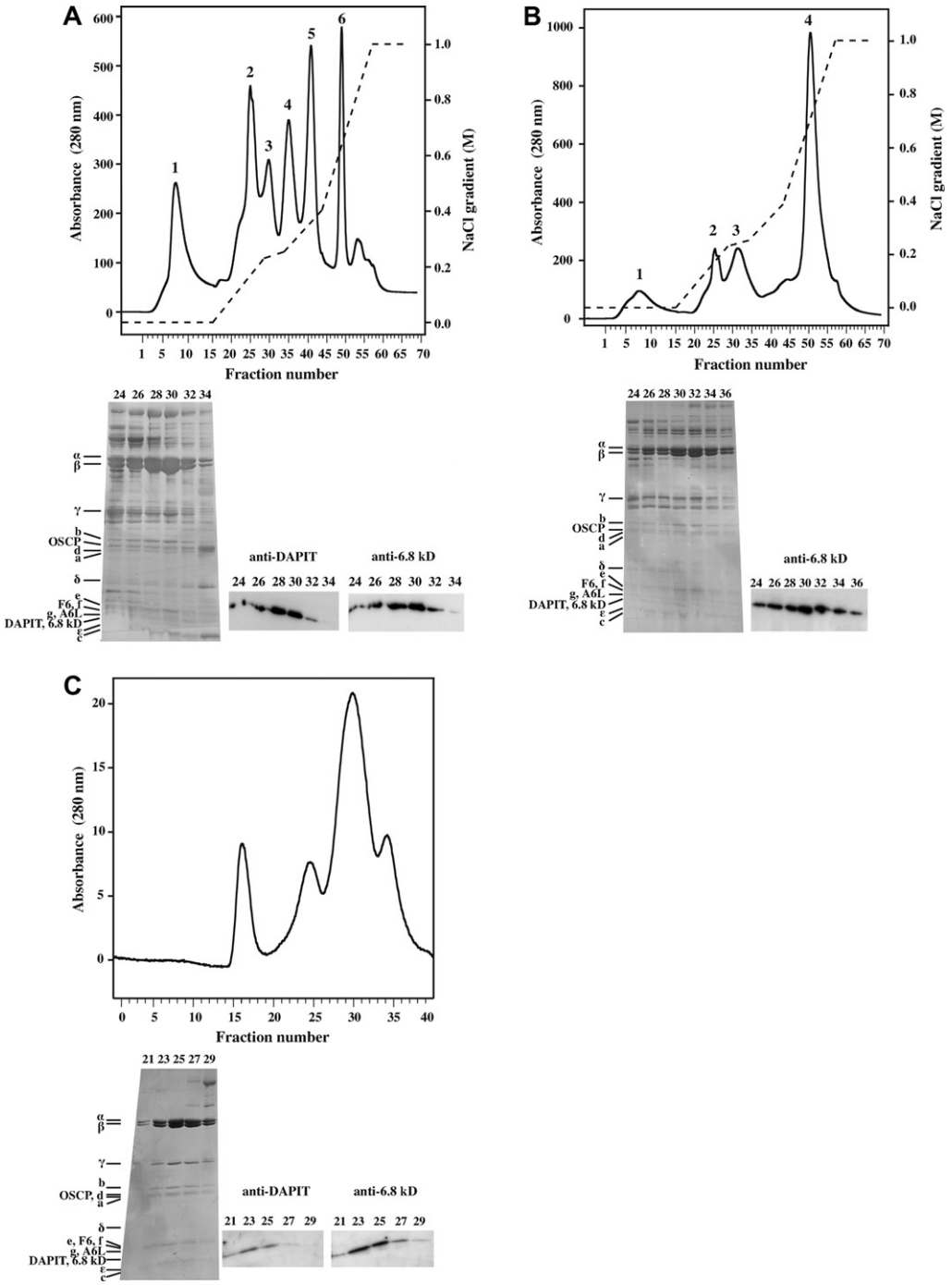

Fig. 1. Co-purification of the 6.8 kDa proteolipid and DAPIT with the F-ATPase from bovine mitochondria. Separation of mitochondrial respiratory protein complexes on Q-Sepharose in the absence (part A) and presence (part B) of exogenous phospholipids. ____, absorbance of the eluant at 280 nm; ---, the sodium chloride gradient to 1 M. In parts A and B, peak 3 contains the F-ATPase. For the identities of other peaks, see the text. Part C, gel filtration chromatography on Superose 6 of F-ATPase from peak 3 in part (B). In parts (A), (B) and (C), the analyses of protein contents by SDS–PAGE followed by staining, of peaks 3 in (A) and (B) and of peak 2 in (C), are shown below the column profiles. The immunodetection of the 6.8 kDa proteolipid and DAPIT are shown to the side. In part B, no attempt was made to detect DAPIT, although as shown in (C), it was clearly present.

and g are removed selectively (M.J. Runswick and J.E. Walker, unpublished results), suggesting that they are less tightly associated with the complex than other subunits. From the present work, the association of the 6.8 kDa proteolipid and DAPIT with the F-ATPase complex solubilised in *n*-dodecyl-β-D-maltoside, which depends on the retention of phospholipids, seems to be even weaker.

Both the bovine 6.8 kDa proteolipid and DAPIT are basic hydrophobic proteins, 60 and 58 amino acids long, respectively, with single transmembrane helical spans. Neither protein has a processed mitochondrial import signal and, other than the removal of the translational initiator methionine residue from DAPIT, neither protein is modified post-translationally [1,2]. Orthologues of both proteins are found in vertebrates, and

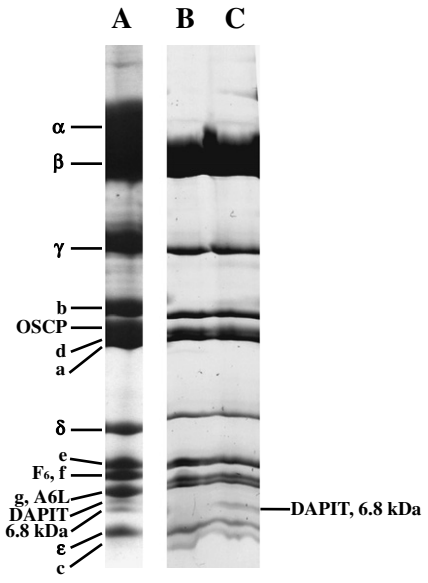

Fig. 2. Subunit compositions of preparations of bovine F-ATPase. Subunits were separated by SDS–PAGE and stained with dye. Lanes (A), (B) and (C), ATP synthase purified by ion exchange and gel filtration chromatography in the presence of exogenous phospholipids, by an affinity purification method in the absence and the presence of exogenous phospholipids, respectively. In (A), the positions of subunits of the F-ATPase and of the 6.8 kDa proteolipid and DAPIT are shown on the left. The 6.8 kDa proteolipid and DAPIT were detected by immuno-blotting and by mass spectrometry (see Supplementary Table 1). In (C), the 6.8 kDa proteolipid and DAPIT, as indicated on the right, were not resolved. They were detected with antibodies.

invertebrates (see Supplementary Material for sequence alignments), but not in *Saccharomyces cerevisiae* and other fungi. The subunit compositions of prokaryotic F-ATPases are simpler than those of eukaryotic enzymes. They are made of an essential core of orthologues of 8 subunits of the mitochondrial enzyme (subunit composition of bacterial F-ATPases, α_3:β_3:γ_1:δ_1:ε_1:a_1:$c_{10\text{ or }11}$:b_2 or alternatively $b_1 : b'_1$, where b and b′ are homologues). Notably, they do not contain orthologues of either mitochondrial subunits e, f, g and A6L, or of the 6.8 kDa proteolipid and DAPIT. Therefore, it is unlikely that any of these proteins participate directly in the formation of ATP. It is more likely that they have other roles for example assisting in the assembly of the complex. It is known, that deletion of subunit e in the yeast enzyme prevents the enzyme from forming dimers in the mitochondrial membrane, and has dramatic effects on the morphology of the inner membranes of the mitochondria [19]. The association of the 6.8 kDa proteolipid and DAPIT in an expression module with proteins involved in oxidative phosphorylation [5] suggests they have significant roles in that process that remain to be defined.

Acknowledgement: This work was supported by the Medical Research Council, UK.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.05.079.

References

[1] Terzi, E., Boyet, P., Van Dorsselaer, A., Luu, B. and Trifilieff, E. (1990) Isolation and amino acid sequence of a novel 6.8-kDa mitochondrial proteolipid from beef heart. Use of FAB-MS for molecular mass determination. FEBS Lett. 260, 122–126.
[2] Carroll, J., Fearnley, I.M. and Walker, J.E. (2006) Definition of the mitochondrial proteome by measurement of molecular masses of membrane proteins. Proc. Natl. Acad. Sci. USA 103, 16170–16175.
[3] Päivärinne, H. and Kainulainen, H. (2001) DAPIT, a novel protein down-regulated in insulin-sensitive tissues in streptozotocin-induced diabetes. Acta Diabetol. 38, 83–86.
[4] Gaster, M. (in press) Insulin resistance and the mitochondrial link. Lessons from cultured human myotubes. Biophys. Biochim. Acta. doi:10.1016/j.bbadis.2007.03.007.
[5] Mootha, V.K., Bunkenborg, J., Olsen, J.V., Hjerrild, M., Wisniewski, J.R., Stahl, E., Bolouri, M.S., Ray, H.N., Sihag, S., Kamal, M., Patterson, N., Lander, E.S. and Mann, M. (2003) Integrated analysis of protein composition, tissue diversity and gene regulation in mouse mitochondria. Cell 115, 629–640.
[6] Smith, A.L. (1967) Preparation, properties and conditions for assay of mitochondria: slaughterhouse material, small scale. Meth. Enzymol. 10, 81–86.
[7] Buchanan, S.K. and Walker, J.E. (1996) Large scale chromatographic purification of F_1F_o-ATPase and complex I from bovine heart mitochondria. Biochem. J. 318, 343–349.
[8] Sazanov, L.A., Peak-Chew, S.Y., Fearnley, I.M. and Walker, J.E. (2000) Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme. Biochemistry 39, 7229–7235.
[9] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[10] Collinson, I.R., Skehel, J.M., Fearnley, I.M., Runswick, M.J. and Walker, J.E. (1996) The F_1F_o ATPase complex from bovine heart mitochondria: the molar ratio of the subunits in the stalk region linking the F_1 and F_o domains. Biochemistry 35, 12640–12646.
[11] Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) Primary structure and subunit stoichiometry of F_1-ATPase from bovine mitochondria. J. Mol. Biol. 184, 677–701.
[12] Walker, J.E., Runswick, M.J. and Poulter, L. (1987) ATP synthase from bovine mitochondria: characterization and sequence analysis of two membrane associated subunits and of their corresponding cDNAs. J. Mol. Biol. 197, 89–100.
[13] Collinson, I.R., Runswick, M.J., Buchanan, S.K., Fearnley, I.M., Skehel, J.M., van Raaij, M.J., Griffiths, D.E. and Walker, J.E. (1994) F_o membrane domain of ATP synthase from bovine heart mitochondria: purification, subunit composition and reconstitution with F_1-ATPase. Biochemistry 33, 7971–7978.
[14] Gibbons, C., Montgomery, M.G., Leslie, A.G.W. and Walker, J.E. (2000) The structure of the central stalk in bovine F_1-ATPase at 2.4 Å resolution. Nat. Struct. Biol. 7, 1055–1061.
[15] Dickson, V.K., Silvester, J.A., Fearnley, I.M., Leslie, A.G.W. and Walker, J.E. (2006) On the structure of the stator of the mitochondrial ATP synthase. EMBO J. 25, 2911–2918.
[16] Collinson, I.R., van Raaij, M.J., Runswick, M.J., Fearnley, I.M., Skehel, J.M., Orriss, G.L., Miroux, B. and Walker, J.E. (1994) ATP synthase from bovine heart mitochondria: *in vitro* assembly of a stalk complex in the presence of F_1-ATPase and its absence. J. Mol. Biol. 242, 408–421.
[17] Stock, D., Leslie, A.G.W. and Walker, J.E. (1999) Molecular architecture of the rotary motor in ATP synthase. Science 286, 1700–1705.
[18] Walker, J.E. and Dickson, V.K. (2006) The peripheral stalk of the mitochondrial ATP synthase. Biochim. Biophys. Acta 1757, 286–296.
[19] Arselin, G., Vaillier, J., Salin, B., Schaeffer, J., Giraud, M.F., Dautant, A., Brèthes, D. and Velours, J. (2004) The modulation in subunits e and g amounts of yeast ATP synthase modifies mitochondrial cristae morphology. J. Biol. Chem. 279, 40392–40399.